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# Methanogenesis and Sulfate Reduction: Competitive and Noncompetitive Substrates in Estuarine Sediments

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Sulfate ions did not inhibit methanogenesis in estuarine sediments supplemented with methanol, trimethylamine, or methionine. However, sulfate greatly retarded methanogenesis when hydrogen or acetate was the substrate. Sulfate reduction was stimulated by acetate, hydrogen, and acetate plus hydrogen, but not by methanol or trimethylamine. These results indicate that sulfate-reducing bacteria will outcompete methanogens for hydrogen, acetate, or both, but will not compete with methanogens for compounds like methanol, trimethylamine, or methionine, thereby allowing methanogenesis and sulfate reduction to operate simultaneously within anoxic, sulfate-containing sediments.

Geochemists and microbiologists have devoted considerable attention to the study of sulfate reduction and methanogenesis. Microbiological investigations indicate that sulfate reducers consume hydrogen, acetate, or both in sulfate-containing sediments and thereby limit the availability of these substrates to methanogens (1, 2, 4, 9, 11, 12, 14, 15, 20, 22–24, 26, 27). This phenomenon helps to explain why a spatial separation is often observed in marine sediments between a zone of sulfate reduction overlying a sulfate-depleted zone of methane production (6, 13). Thus, despite some observations to the contrary (10, 14, 20), sulfate reduction is thought to preclude methanogenesis.

However, it was recently reported that methanogenesis in Big Soda Lake, Nev., sediments (sulfate, ≅68 mM) was greatly stimulated by addition of methanol, trimethylamine, or methionine but not by hydrogen, acetate, or formate (17). Furthermore, we observed that trimethylamine and methanol can be important substrates for methanogenic bacteria in salt marsh sediments and that conversion of methanol to methane was unaffected by the presence or absence of sulfate ions (18). In addition, other workers have noted a rapid conversion of [$^{14}$C]methylamine to [$^{14}$C]methane in sulfate-containing marine sediments (M. Winfrey and D. Ward, personal communication; manuscript submitted). Thus, methanogenesis may take place in sulfate-containing marine sediments by catabolism of substrates other than hydrogen or acetate. In this paper, based on experiments with substrate-supplemented sediment slurries and an enrichment culture of a sulfate-reducing bacterium (SRB), we report that sulfate reducers outcompete methanogens for hydrogen and acetate, but do not compete with methanogens for methanol, trimethylamine, or methionine.

## MATERIALS AND METHODS

**Experiments with sediment slurries.** Sediments were collected during 1980–1981 from an intertidal mudflat located in south San Francisco Bay (16). Sediment cores were homogenized anaerobically and dispensed into 125-cm$^3$ Erlenmeyer flasks by procedures outlined previously (16). However, instead of using San Francisco Bay water to generate slurries, a mineral salts solution was employed. The mineral salts solution had the following composition (grams per liter of distilled water): NaCl, 12; $MgCl_2 \cdot 6H_2O$, 5.5; $CaCl_2 \cdot 2H_2O$, 0.75; KCl, 0.38; NaBr, 0.04; $Na_2SO_4$ or NaCl, 3.0; $NaHCO_3$, 0.25; and trace elements solution (30), 10 ml/liter. The pH was adjusted to 7.0. SRBs were therefore inhibited by incubation in mineral salts medium that lacked sulfate ions. This procedure was chosen instead of molybdate inhibition of SRBs (4, 7, 20, 23, 24), because molybdate forms a complex with free sulfide ions (4, 19, 29) and may therefore limit growth of methanogens due to a lack of available sulfide (5). Sediment cores (volume, ≅400 cm$^3$; upper 30 cm of sediment column) were first homogenized in the sulfate-free mineral salts solution (sediment-mineral salts, 1:1 [vol/vol]), and the resulting homogenate (25 ml) was dispensed into flasks containing 50 ml of either the sulfate-free or sulfate-containing mineral salts solution. Some interstitial sulfate was probably carried over into the sulfate-free flasks by this procedure; however, it was a minor amount. Assuming an average core water content of 50% and an average interstitial sulfate concentration of ~10 mM, the maximum amount of carry-over sulfate would be ~60 μmol per flask or about 6% of the sulfate-containing flasks (1.06 mmol per flask). Thus, in this paper, slurries referred to as sulfate free actually contained small quantities of sulfate (<60 μmol per flask), which would quickly be depleted by SRBs compared with sulfate-containing flasks.

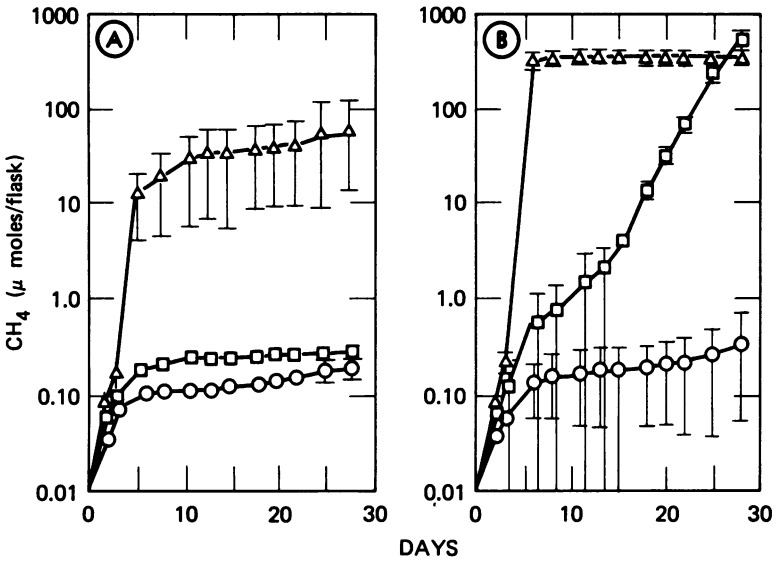


FIG. 1. Production of $CH_4$ by sediment slurries incubated in the presence (A) and absence (B) of sulfate. Slurries were supplemented with $H_2$ (△), acetate (□), or nothing (○). Points represent the mean of three flasks; bars indicate one standard deviation.

In some experiments, larger Erlenmeyer flasks were used, with proportionate increases of the slurry and gas-phase volumes. Flasks were incubated in the dark (20°C) with constant rotary shaking (200 rpm) under an atmosphere of either $N_2$ or $H_2$. Uptake of $H_2$ due to bacterial consumption was measured by equilibration with $H_2$-filled glass syringes. Additions were made to selected flasks (125-$cm^3$ Erlenmeyer) of sodium acetate, sodium formate, DL-methionine, methanol, or trimethylamine (10 mM; 500 μmol per flask). 2-Bromoethanesulfonic acid (BES), a specific inhibitor of methanogenic bacteria (8, 16), was added where indicated to inhibit methanogenesis (5 mM; 250 μmol per flask).

**Determination of $CH_4$, $CO_2$, and sulfide.** $CH_4$ and $CO_2$ in the gas phases of flasks were measured by gas chromatography (7, 16). Formation of sulfide was measured by $N_2$ stripping of acidified sediment or SRB culture samples, trapping the evolved $H_2S$ as CdS, and analyzing the redissolved $Cd^{2+}$ by atomic absorption spectroscopy (19).

**Enrichment cultures.** An enrichment culture of an SRB capable of growth upon $H_2$-$CO_2$-acetate was obtained from sediment slurries incubated under $H_2$ with acetate. The enrichment medium was composed of (grams per liter of distilled water): NaCl, 20; $Na_2SO_4$, 3.0; $NH_4Cl$, 1.0; $MgSO_4 \cdot 7H_2O$, 2.0; $KH_2PO_4$, 0.25; $CaCl_2 \cdot 2H_2O$, 0.2; $K_2HPO_4$, 0.25; sodium acetate, 5.0; and $FeSO_4 \cdot 7H_2O$, 0.25. It also contained (per liter): resazurine, 1 mg; trace elements solution (28), 10 ml; $NaHCO_3$, 0.25 g; $Na_2SO_3$, 2.8 g; and vitamin solution (28), 10 ml. The $NaHCO_3$, $Na_2SO_3$, and vitamin solution were added by filter sterilization (0.22 μm; Millex) of stock solutions after the autoclaved medium (15 $lb/in^2$, 20 min) was allowed to cool in 250- or 500-$cm^3$ Erlenmyer flasks (100 or 200 ml of medium per flask, respectively). Flasks were sealed with recessed black rubber stoppers and flushed for 10 min with sterile, $O_2$-free $N_2$-$CO_2$ (4:1; ~100 ml/min). The final pH of the medium was 7.0. After inoculation (5 ml of slurry from a sediment slurry incubated under $H_2$-acetate for 2 weeks) or transfer of cultures (5 ml by syringe at 2 to 3 weeks), the gas phase was changed to $H_2$-$CO_2$ (4:1) by flushing. Flasks were incubated in the dark (20°C) with constant rotary shaking (200 rpm). Uptake of $H_2$-$CO_2$ was monitored by equilibration with $H_2$-$CO_2$-filled glass syringes fitted with sterile filters (0.22 μm). Sulfide and $CH_4$ were measured as indicated above. During the initial incubation, BES (2 mM) was included in the medium to retard the growth of methanogens. BES was eliminated from the medium in all subsequent transfers. To test whether the enrichment required $H_2$ or acetate for growth (sulfide production), media were prepared both with and without acetate and, after inoculation, incubated under $N_2$ or $H_2$. In another experiment, the effects of including molybdate (20 mM) in or eliminating $SO_4^{2-}$ and $SO_3^{3-}$ from the medium on uptake of the $H_2$-$CO_2$ gas mixture were tested.

## RESULTS

**Sediment slurries.** Sediment slurries produced methane (usually without a time lag) and production was stimulated by the presence of either hydrogen or acetate (Fig. 1A and B). Sulfate ions greatly retarded the rate and extent of methanogenesis from these substrates. After 4 weeks of incubation in the absence of sulfate, hydrogen and acetate caused 1,170-fold and 2,000-fold enhancement of methanogenesis, respectively, as compared with unsupplemented slurries (Fig. 1B). By contrast, slurries incubat-

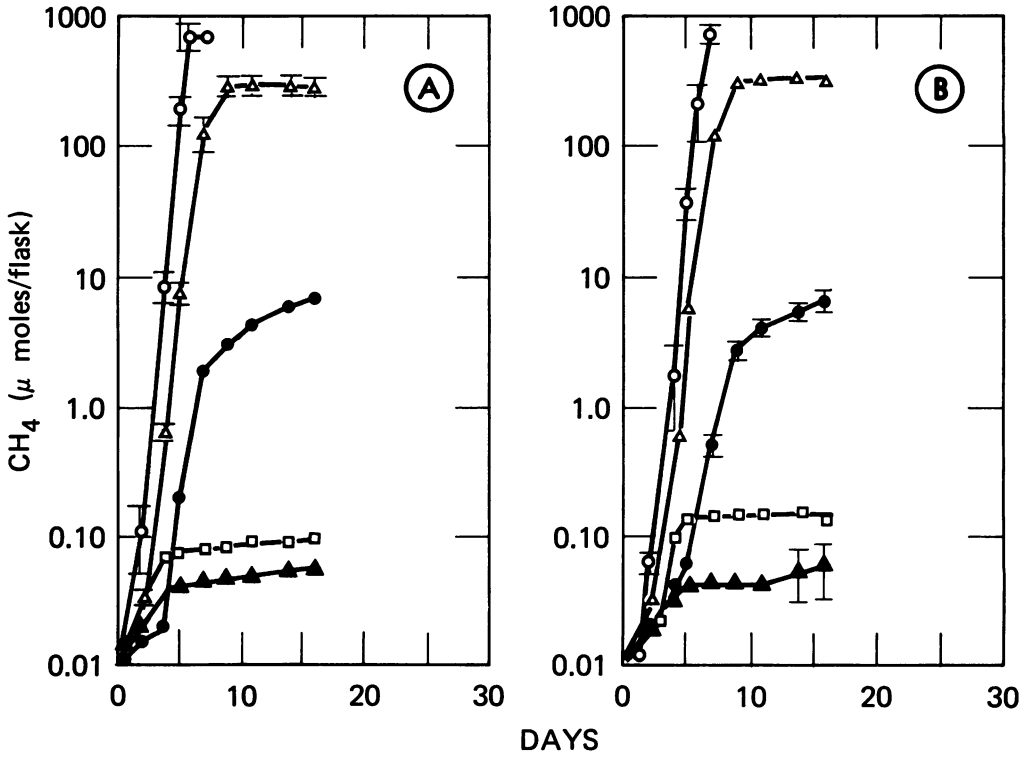


FIG. 2. Production of $CH_4$ by sediment slurries incubated in the presence (A) and absence (B) of sulfate. Symbols: slurries supplemented with trimethylamine (○), methanol (△), methionine (●), formate (□), or no additions (▲). Points represent the mean of three flasks; bars indicate one standard deviation. Experiments were conducted during March 1981, except for trimethylamine (July 1981).

ed for 4 weeks in the presence of sulfate were stimulated only 200-fold and 1.5-fold by hydrogen and acetate, respectively (Fig. 1A).

Addition to the slurries of trimethylamine, methanol, or methionine caused extensive enhancement of methanogenesis, and results were nearly identical for slurries incubated either with or without sulfate (Fig. 2A and B). The absence

TABLE 1. Effect of sulfate and BES on methane formation from various compounds

| Addition (mo/yr) | $CH_4$ (μmol per flask ± SD)[a] | | |
|---|---|---|---|
| | With sulfate | Without sulfate | BES[b] |
| None (3/81) | 0.06 ± 0.008 | 0.06 ± 0.002 | 0.02 ± 0.002 |
| Formate (3/81) | 0.10 ± 0.006 (0.03) | 0.12 ± 0.004 (0.05) | 0.04 ± 0.004 |
| Methionine (3/81) | 6.8 ± 1.4 (1.4) | 6.6 ± 1.2 (1.3) | 0.04 ± 0.002 |
| Methanol (3/81) | 306 ± 20 (82) | 294 ± 28 (78) | 0.02 ± 0 |
| Trimethylamine (7/81) | 752 ± 32 (67) | 780 ± 94 (69) | ND |
| Acetate (3/80) | 0.27 ± 0.03 (0.1) | 15 ± 6.4 (3) | ND |
| Acetate (7/81) | 8.5 ± 0.3 (1.7) | 200 ± 13 (40) | 0.11 ± 0.01 |
| $H_2$[c] (3/80) | 39 ± 31 (3.5) | 369 ± 59 (64) | ND |
| $H_2$[d] (7/81) | 358 ± 58 (28) | 500 ± 46 (86) | 0.18 ± 0.01 |

[a] Values represent the mean of three flasks. Incubation was for 16 to 18 days, except for trimethylamine (7 days). All soluble substrates were used at a concentration of 10 mM (500 μmol per flask). Percent conversion efficiencies (in parentheses) are based on the following reactions: 4 $HCOOH \rightarrow CH_4 + 3CO_2 + 2H_2O$; 4 methionine → 4 homoserine + $3CH_4 + HCO_3^- + 4H_2S + H^+$; 4 $(CH_3)_3N + 6H_2O \rightarrow 9CH_4 + 3CO_2 + 4NH_4^+$; $4CH_3OH \rightarrow 3CH_4 + CO_2 + 2H_2O$; $4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$; and $CH_3COOH \rightarrow CH_4 + CO_2$.

[b] BES flasks contained sulfate. ND, Not determined.

[c] $H_2$ uptake was 4.5 and 2.3 mmol in media with and without sulfate, respectively, after an 18-day incubation.

[d] $H_2$ uptake was 5.2, 2.3, and 2.0 mmol in media with sulfate, without sulfate, and with BES, respectively, after a 16-day incubation.

TABLE 2. Effect of substrate additions on sulfate reduction (production of sulfide) by sediment slurries

| Addition | Sulfide (μmol per flask)[a] | |
|---|---|---|
| | Expt 1 | Expt 2 |
| None | 243 | 125 |
| Acetate | 808 | 498 |
| $H_2$[b] | 1,224 | 1,134 |
| $H_2$ plus acetate[c] | 1,786 | 2,094 |

[a] Experiment 1 began on 24 October 1980, and experiment 2 began on 11 December 1980. Slurries (300-ml volume) were incubated in 500-ml Erlenmeyer flasks for 14 days (experiment 1) or 16 days (experiment 2).

[b] $H_2$ uptake was 7.2 mmol in experiment 1 and 10.0 mmol in experiment 2.

[c] $H_2$ uptake was 8.0 mmol in experiment 1 and 14.6 mmol in experiment 2.

of sulfate actually caused a slight depression in the rate of methanogenesis from these substrates. Formate caused only a 1.7-fold and 2.0-fold stimulation of methanogenesis in slurries incubated in the presence and absence of sulfate, respectively.

Table 1 summarizes the effect of sulfate on methanogenesis from the various substrates and indicates their percent conversion to methane. The percent conversions of methionine, methanol, and trimethylamine were essentially identical both in the presence and absence of sulfate, and with methanol and trimethylamine the conversions were high (67 to 82%). By contrast, acetate and hydrogen had much lower conversion efficiencies with sulfate than without it. However, methanogenesis from hydrogen or acetate was much more extensive and efficient during July 1981 than in March 1980, implying that a strong seasonality is associated with the potential for methanogenic activity in these sediments. Formate caused only a slight enhancement of methanogenesis, and because its percent conversion to methane was so low (0.03 to 0.05%), it appears to be unimportant as a precursor of methane. BES inhibited methane formation from all substrates tested. The effect of BES on methane formation from trimethylamine was not tested in these sediments. However, BES proved to be effective at blocking trimethylamine conversion to methane in sediments from a neighboring salt marsh (18) and from Big Soda Lake (17).

The production of sulfide by slurries was stimulated by addition of acetate, hydrogen, and hydrogen plus acetate (Table 2). By contrast, the addition of methanol or trimethylamine provided no stimulation of sulfate reduction (sulfide formation) over that observed in slurries incubated without substrate additions (Table 3). The effect of methionine on sulfate reduction was not tested because $H_2S$ and $CH_3SH$ are formed during its decomposition (31) and would therefore mask formation of $H_2S$ by the SRB.

Figure 3 shows the levels of methane and carbon dioxide and the uptake of hydrogen by slurries incubated under an $H_2$ atmosphere. The slurries were divided into three experimental groups: (i) no additions, (ii) supplemented with sulfate, and (iii) supplemented with sulfate and acetate. Methane production commenced after a lag of 4 days in the slurries incubated without sulfate (Fig. 3A). Longer lag periods were evident in the slurries containing sulfate (7 days) and sulfate plus acetate (15 days). Carbon dioxide disappeared from the gas phases of all of the flasks after 7 days of incubation (Fig. 3B). The disappearance of carbon dioxide was accelerated by sulfate ions and was most rapid in the flasks containing sulfate plus acetate. By contrast, slurries incubated under $N_2$ atmospheres (either with or without substrate or sulfate additions) always produced $CO_2$ (data not shown). Slurries containing sulfate or sulfate plus acetate consumed 2.5 and 2.3 times more $H_2$, respectively, than did slurries incubated without sulfate (Fig. 3C [day 19]). In another experiment, the effects of 0, 20, and 40 mM sulfate on methanogenesis and $H_2$ consumption were followed. After 2 weeks of incubation, slurries supplemented with 0, 20, and 40 mM sulfate consumed 2 ± 0.02, 5.4 ± 0, and 8.0 ± 0.25 mmol of $H_2$ and produced 348 ± 25, 182 ± 8, and 24 ± 8 μmol of $CH_4$, respectively (mean of three flasks ± one standard deviation). After 4 weeks of incubation, $H_2$ consumed increased to 2.4 ± 0, 6.6 ± 0.05, and 10.3 ± 0.17 mmol and $CH_4$ increased to 346 ± 38, 347 ± 12, and 252 ± 9 μmol for the 0, 20, and 40 mM sulfate flasks, respectively.

**Enrichment cultures.** The enrichment culture of the SRB consumed the $H_2$-$CO_2$ gas mixture during incubation (8.7 mmol by day 25). Uptake of the gas mixture was entirely inhibited by

TABLE 3. Effect of substrate additions on sulfate reduction (production of sulfide) by sediment slurries[a]

| Addition | Sulfide (μmol per flask ± SD) | $CH_4$ (μmol per flask ± SD) |
|---|---|---|
| None | 66 ± 1.7 | 0.03 ± 0.009 |
| Methanol | 68 ± 7.5 | 98 ± 25 |
| Trimethylamine | 66 ± 10.4 | 377 ± 75 |

[a] Experiment was run during June 1981. Slurries (75-ml volumes) were incubated for 6 days in 125-$cm^3$ Erlenmeyer flasks. Values represent the mean of three flasks.

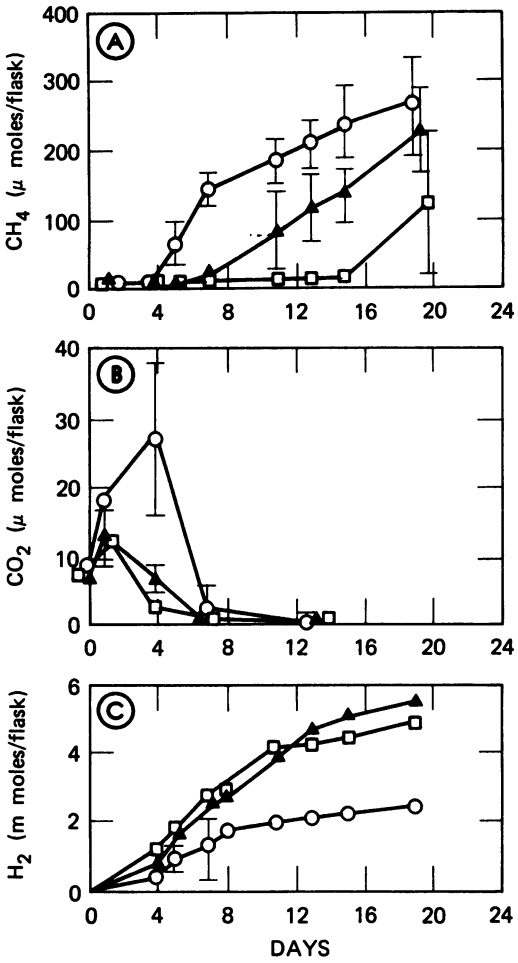


FIG. 3. Levels of $CH_4$ (A) and $CO_2$ (B) in the gas phases of flasks containing sediment slurries. All slurries were incubated under a hydrogen atmosphere and developed negative pressures. (C) Consumption of $H_2$ by these sediment slurries. Symbols: flasks containing slurries incubated in mineral salts medium without sulfate (○), with sulfate (▲), and with sulfate plus acetate (□).

molybdate ions or by elimination of sulfate and sulfite ions from the medium. No significant levels of $CH_4$ were observed in the gas phases of the enrichment. However, $CO_2$ disappeared from the gas phases of flasks within 1 day after addition of $H_2$-$CO_2$. Production of sulfide by the enrichment was stimulated by $H_2$, but more extensive stimulation occurred when the enrichment was incubated with $H_2$-acetate. Over a 2-week period, the $H_2$ plus acetate enrichment produced sulfide at a rate of ~300 μmol per day, compared with a rate of ~130 μmol per day for the enrichment incubated with $H_2$ but without acetate. No sulfide was produced under $N_2$ with or without acetate. The amount of sulfide formed in the flask with $H_2$ and acetate (≅4.5 mmol of $S^{2-}$ by day 15) represented ~50% conversion of the available sulfate and sulfite ions (8.8 mmol/200 ml of medium) to sulfide. By contrast, the total amount of sulfide formed by the $H_2$-without-acetate flask was only ~1.75 mmol of $S^{2-}$. Examination of the enrichment culture by phase-contrast microscopy (Nikon Optiphot; 1,000×) revealed the predominant organism to be a vibrio growing either singly or in chains of two to three cells. Pairs of attached cells had a distinctive seagull appearance morphologically similar to *Desulfovibrio gigas* (21).

## DISCUSSION

In this report we have shown that SRBs will outcompete methanogens for $H_2$, acetate, or both. Results of the sediment slurry experiments clearly indicate that sulfate inhibits methane production from $H_2$ and acetate (Fig. 1A and B; Table 1; Fig. 3A). The stimulation of sulfate reduction by acetate, $H_2$, and acetate plus $H_2$ (Table 2) and recovery of an enrichment culture of an SRB capable of growth on $H_2$-acetate-$CO_2$ further reinforces the notion that, given a supply of sulfate ions, sulfate reducers will outcompete methanogens for the primary methanogenic substrates (e.g., $H_2$ and acetate). The inhibition of methanogenesis observed when slurries were supplemented with $H_2$, acetate, and sulfate (Fig. 3A) was therefore probably due to the growth of a sulfate reducer similar to the one recovered in our enrichment culture. At first glance this result is perplexing because methanogens should thrive when provided with two important substrates ($H_2$ and acetate). However, these conditions may favor sulfate reduction, especially if the SRBs have a lower $K_m$ for $H_2$ (12) and acetate than the methanogens do. Since hydrogen may become limiting during these incubations (because of low-phase transfer and intervals of reduced $H_2$ partial pressure within the flasks), it is not unreasonable to invoke competitive exclusion as a mechanism for inhibition of methanogenesis by sulfate. These results come as no surprise considering the numerous observations of sulfate reducers outcompeting methanogens for these substrates in sediments (1, 2, 4, 7, 9, 11, 12, 14, 15, 20, 22–24, 28) and the isolation of sulfate reducers capable of growth on acetate (26, 27) or on the combination of acetate $H_2$, and $CO_2$ (3, 25). This confirms what was established with molybdate inhibition experiments (20, 24), that more $CH_4$ is formed when SRBs are blocked by molybdate. In earlier studies of sulfate reduction in these sediments, Oremland and Silverman (19), employing an electrical impedance technique, did not note any enhancement of sulfate reduction by acetate addition. Since the electrical impedance tech-

nique responds to the deposition of FeS particles on the electrode surface, perhaps only intensive stimulation of sulfate reduction, such as that caused by lactate, was capable of eliciting an electrode response.

The surprising results of these experiments are that sulfate ions do not retard methane production from methanol, trimethylamine, or methionine (Fig. 2A and B). Furthermore, since neither methanol nor trimethylamine stimulated sulfate reduction, and because these two compounds had high conversion efficiencies to methane (Table 1), it appears that methanogens are the dominant group of microbes responsible for the anaerobic decomposition of these substrates. These results, therefore, reinforce observations made in a neighboring salt marsh where we noted the accumulation of methanol and trimethylamine in sediments inhibited with BES (18).

With methionine, conversion to methane appears to proceed via the intermediate methylmercaptan (31). The observed low conversion efficiencies of 1.3 to 1.4% (Table 1) may therefore reflect slow reactions leading to the formation of the intermediate rather than the methane end product. Nevertheless, since our results for methionine were qualitatively the same as for methanol and trimethylamine (no inhibition by sulfate), the low efficiency of conversion suggests that the methylmercaptan intermediate is also primarily attacked by methanogens and not by SRB. The formation of methane from a methionine decomposition intermediate (methylmercaptan) was due to methanogens, since BES inhibited methane formation from methionine (Table 1). Inhibition of methane formation from methionine and methylmercaptan was also reported for lake sediments incubated with $CHCl_3$ (31, 32). Zinder and Brock (32), however, did not observe the conversion of methylmercaptan to methane by pure cultures of methanogenic bacteria. Since the coenzyme M analog BES proved to be effective at blocking methanogenesis from methionine in our estuarine sediments, perhaps a yet-to-be-isolated methanogen resides in aquatic sediments which can form methane from methylmercaptan or some other methionine degradation product.

It is therefore clear that methanogenesis and sulfate reduction can occur simultaneously in anoxic, sulfate-rich sediments provided there is an adequate supply of noncompetitive substrates (e.g., methanol, methylamines, methionine) or an abundance of competitive substrates (e.g., $H_2$ or acetate) for the methanogenic bacteria to utilize. Preliminary observations in salt marsh sediments favor the former (18). Future research, therefore, aimed at delineating pathways and rates of formation of these noncompetitive methanogenic precursors and their pool sizes, turnover times, and relative importance vis-a-vis the competitive precursors ($H_2$ and acetate) should prove to be an interesting area for future endeavor.

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